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Studies of Astaxanthin Biosynthesis in *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Effect of Inhibitors and Low Temperature

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The effect of nicotine and diphenylamine on astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous* was studied. The effects were analysed under standard and low temperature conditions. It was found that 10 mM-nicotine inhibits the cyclization of lycopene and *de novo* protein synthesis was not needed to reverse the inhibition. The oxidation of β-carotene was irreversibly inhibited by 10 μM-diphenylamine while the dehydrogenation of phytoene was reversibly inhibited by 60 μM-diphenylamine. The simultaneous exposure to low temperature (4°C) overcomes the inhibition of β-carotene oxidation at low diphenylamine concentration. © 1998 John Wiley & Sons, Ltd.

KEY WORDS — nicotine; diphenylamine; astaxanthin biosynthesis; *Xanthophyllomyces dendrorhous*

INTRODUCTION

Xanthophyllomyces dendrorhous (Golubev, 1995), previously named *Phaffia rhodozyma*, is a moderately psychrophilic yeast, with a maximum growth temperature of approximately 27°C (Miller *et al.*, 1976). Wild-type strains produce astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), a potent antioxidant, whose activity against peroxide radical-mediated phospholipid peroxidation may be stronger than that of β-carotene (Lim *et al.*, 1992). Presently the main demand for astaxanthin comes from aquaculture farmers, who need it to supplement the food source in order to achieve normal pigmentation of the fish flesh in salmon and other economically important species (Johnson *et al.*, 1977).

The biosynthetic pathway of astaxanthin from β-carotene was determined in the green alga *Haematococcus pluvialis* using diphenylamine as an inhibitor (Fan *et al.*, 1995) and in *Agrobacterium aurautiacum* under different culture conditions. In both cases, cantaxanthin was an intermediate, and depending on the availability of oxygen, *A. aurautiacum* has an alternative pathway with zeaxanthin as intermediate. The genes for the carotenogenic enzymes from *A. aurautiacum* were cloned and it was demonstrated that two different genes are involved in the conversion of β-carotene to astaxanthin (Misawa *et al.*, 1995).

As schematically shown in Figure 1 two alternative pathways were postulated in *X. dendrorhous*. Both of them imply that first by an asymmetric oxidation at C-4 in one of the rings in β-carotene and in the single ring in torulene, a keto group is formed, while oxidation at the second C-4 in β-carotene to a keto group is preceded by the hydroxylation at the C-3 on the same ring, yielding the intermediaries 3-OH-echinenone or 3-hydroxy-3′,4′-didehydro-β-ψ-carotene-4-one (HDCO) from β-carotene and torulene, respectively. In contrast to *H. pluvialis* and *A. aurautiacum*, neither cantaxanthin nor zeaxanthin were found in *X. dendrorhous* under any culture conditions studied (Andrewes *et al.*, 1976; Johnson and Lewis, 1979).

Temperature is one of the most important environmental factors that affect all aspects of growth and development of living organisms. The effects range from polysaccharide and pigment synthesis to flagella production, sporulation, as well as the composition and integrity of the cell membrane and wall structures, and include changes in the fatty acid profile of cellular lipids (Suutari and Laakso, 1994). Therefore it may be expected that changes in temperature also induce changes in carotenogenesis.

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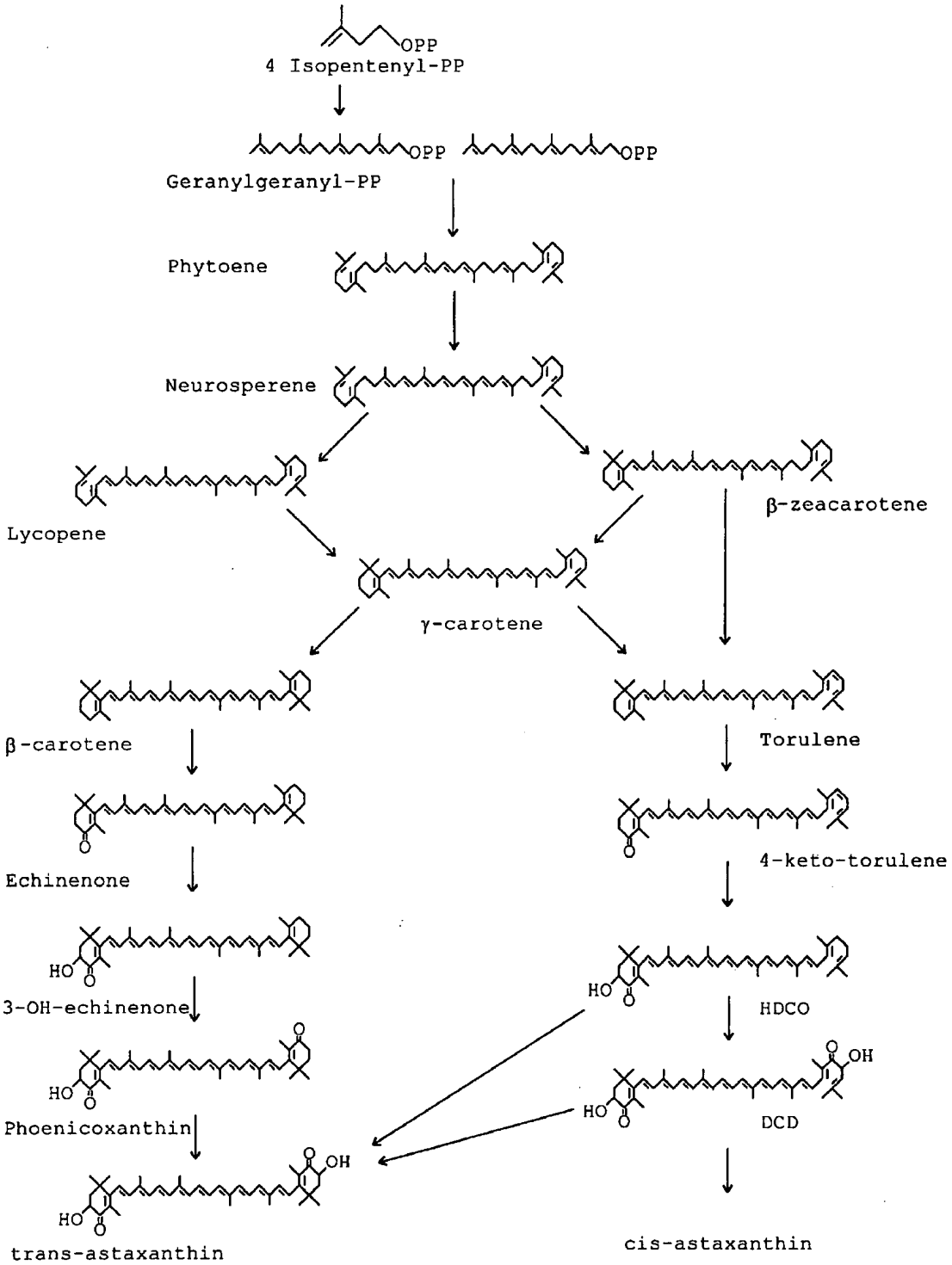

Figure 1. Presumed pathway of astaxanthin biosynthesis in *X. dendrorhous*.

Here we studied in detail the inhibition of carotenogenesis in the yeast *X. dendrorhous* by nicotine and diphenylamine at optimal (22°C) and low temperature (4°C) conditions. Nicotine is known to inhibit the cyclizing steps (Howes and Batra, 1970) in β-carotene synthesis, while diphenylamine leads to a block in the desaturation of phytoene (Clarke *et al.*, 1983).

MATERIALS AND METHODS

Strain

Xanthophyllomyces dendrorhous NRRL Y-17269 was obtained from the NRRL collection in Peoria, Illinois, by courtesy of Dr C. P. Kurtzman. *Xanthophyllomyces dendrorhous* mutant 5-7 (β-carotene accumulating strain) was isolated by UV mutagenesis (Ducrey, 1995).

Media

X. dendrorhous was grown at 22°C on YM medium containing (in %, w/v) yeast extract 0·3, malt extract 0·3, peptine 0·5, glucose 1·0; pH was adjusted to 4·5–5·5 using 1·0 N-HCl. For solid media 1·5% agar was added. Cultures in liquid medium were grown in conical flasks (500 ml) with shikanes containing 10% (v/v) of the broth and agitated on a rotary shaker at a frequency of 3·3 Hz.

Chemicals

All the solvents used were of HPLC grade (Fisher Scientific, Leicestershire, UK or Carl Roth GmbH, Karlsruhe, Germany) and the other reagents were of analytical grade obtained from Merck, Darmstadt, Germany, except for (−)-nicotine (Carl Roth GmbH).

Carotenoid extraction and quantitative determination

Carotenoids were extracted with dimethylsulfoxide and acetone according to Sedmak *et al.* (1990) and transferred to petroleum ether. When the strains produced astaxanthin, total carotenoids were calculated as described by An *et al.* (1989) using a 1% absorption coefficient of 2100 and the maximal peak absorbance value at 474 nm. When the strains produced β-carotene or lycopene, 1% absorption coefficients of 2500 (450 nm) and 3450 (470 nm), respectively, were used (Britton, 1995). Phytoene concentration was determined using the absorption coefficient of 1250 and the maximal peak absorbance value at 286 nm (Britton, 1995). Absorption spectra were determined on a Beckman DU650 spectrophotometer (Fullerton, USA). β-carotene and lycopene were identified by TLC and HPLC in comparison with standards. *Trans-* and *cis*-astaxanthin, phoenicoxanthin, hydroxyechinenone, echinenone and phytoene were identified by their chromatographic properties and absorption spectra as indicated by Andrewes *et al.* (1976) and Britton (1995).

Individual carotenoid concentrations were determined by HPLC analysis, carried out on a Gynkotech device (Germering, Germany) with a high precision pump (model 300 B), an automatic injector (Gina 169), and integrator (C-R6A) and an UV-visible adsorption detector (SP-6), using a reversed-phase C18 column (Hypersil ODS5) and a mobile phase of methanol-acetonitrile (9 : 1). The separation was carried out at ambient temperature. Individual carotenoids were also analysed by TLC on silica gel (Kieselgel 60, thickness 0·2 mm, Merck) using a mobile phase containing petroleum ether-acetone (2 : 1) according to Fan *et al.* (1995).

Lycopene and β-carotene purchased from Sigma-Aldrich GmbH (Germany) were used as standards to construct calibration curves.

Microbial growth

Biomass dry weight was determined at 105°C after separating the cells by centrifugation (10,000 *g*, 10 min) followed by washing with distilled water and resedimentation.

RESULTS

Effect of inhibitors on growth

In order to avoid any non-specific effect of diphenylamine and nicotine, we determined first which concentration has an inhibitory effect on the carotenogenesis but not a deleterious effect on the growth of *X. dendrorhous*. Cells were grown under optimal conditions in the presence or absence of both inhibitors. In Table 1 we show the dry weight and total carotenoid concentration as a function of the inhibitor concentration. These results show that nicotine had almost no negative effect on growth at concentrations as high as 20 mM, as observed also by Girard *et al.* (1994), while diphenylamine inhibited growth progressively at concentrations higher than 60 μM.

Table 1. Biomass, phytoene and total carotenoid concentrations in a *X. dendrorhous* culture as a function of diphenylamine and nicotine concentration.

		Inhibitor concentration										
		Diphenylamine (μM)								Nicotine (mM)		
	Control	5	10	20	30	60	90	120	240	5	10	20
Dry weight (mg/ml)	4·3	4·7	4·4	4·7	4·5	5·1	3·6	1·1	0·2	3·5	3·6	3·5
Total carotenoids (μg/g)	340	ND	36J	140	130	22	0	0	0	360	350	300
Phytoene (μg/g)	2540	ND	1590	ND	1770	2890	ND	ND	ND	ND	4000	3300

Control: without inhibitor.
ND: not determined.

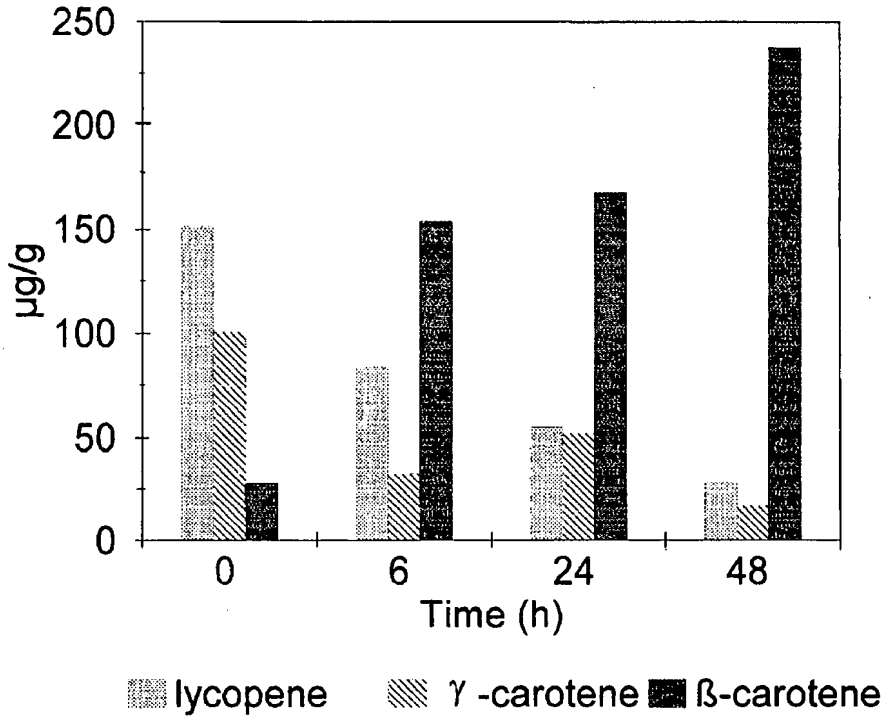

Figure 2. Carotenoid composition (μg/g biomass) of mutant 5-7 grown in the presence of nicotine (10 mM). Time 0 before removal of the inhibitor by a washing step, Times 6, 24 and 48: hours of incubation after removal of the inhibitor. Incubation temperature: 22°C.

Effect of nicotine on carotenogenesis

Nicotine is known to specifically inhibit the cyclization steps in carotenogenesis (Shaish *et al.*, 1990). In our studies the presence of 10 mM-nicotine in cultures of mutant 5-7 (β-carotene accumulating) leads to the accumulation of lycopene, the most saturated open carotenoid, but also considerable amounts of γ-carotene are observed. This implied an inhibition of the lycopene cyclase synthesizing β-carotene from lycopene, via γ-carotene as intermediate (Girard *et al.*, 1994). If the nicotine is removed by several washes the normal flow of metabolites in the carotenogenesis is restored with a decrease in lycopene concentration and a subsequent accumulation of β-carotene, and a decrease in γ-carotene (Figure 2).

Incubation of wild-type *X. dendrorhous* with nicotine led to the same results as observed with mutant 5-7, but instead of increasing β-carotene, astaxanthin is found after nicotine removal as a

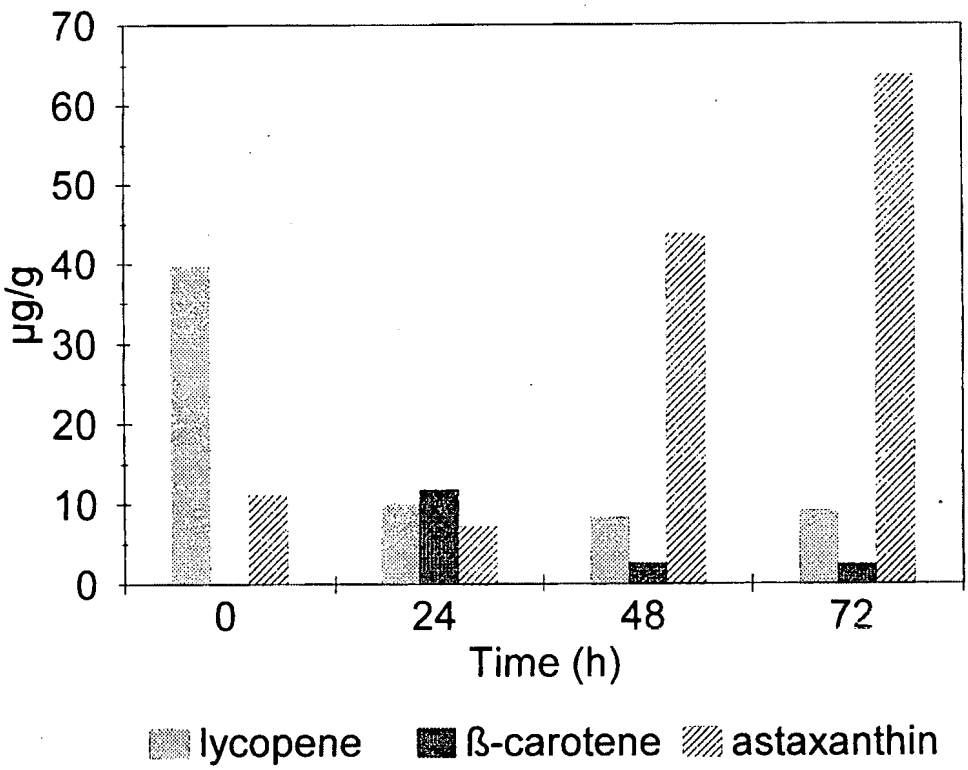

Figure 3. Carotenoid composition (µg/g biomass) of wild-type *X. dendrorhous* grown in the presence of nicotine (10 mM). Time 0: before removal of the inhibitor by a washing step. Times 24, 48 and 72: hours of incubation after removal of the inhibitor. Incubation temperature: 22°C.

final product as well as its oxygenated precursors (xanthophylls) (Figure 3).

The total carotenoid concentration, as measured by visible absorption, did not change significantly in the presence of inhibitors (Table 1), while the colourless intermediate phytoene is accumulated in rather high amounts in the presence of nicotine.

Addition of cycloheximide to the culture, blocking translation, did not prevent the cyclization reaction after the removal of nicotine, neither in the mutant 5-7, nor in the wild-type strain.

Effect of diphenylamine on carotenogenesis

Diphenylamine did not have a deleterious effect on *X. dendrorhous* in concentrations up to 60 µM. At low concentrations (10 µM) oxidation of β-carotene was inhibited (Figure 4a) but at higher concentrations the amount of accumulated β-carotene decreased until the desaturation of phytoene was completely inhibited (60 µM-diphenylamine) yielding colourless cells (Figure 4c). The normal pigmentation reoccurred after washing the cells. The presence of cycloheximide after the washing step prevented the conversion of β-carotene into astaxanthin (Figure 4b and d). However, incubating *X. dendrorhous* in the presence of diphenylamine led not only to the inhibition of different steps in the biosynthetic pathway but also decreased the total concentration of carotenoids, even when phytoene is included in the total sum. When the formation of β-carotene was blocked (60 µM of diphenylamine), the amount of phytoene increased compared to the control (Table 1).

Effect of low temperatures and inhibitors on carotenogenesis

Low temperatures cause changes in the composition and concentration (50% increase) of total carotenoids in *X. dendrorhous* (Ducrey, 1995) and we tested what happens when cells under the pressure of carotenogenic inhibitors are exposed to low temperature (4°C). First, cells were grown at 22°C in the presence of inhibitor, then the culture

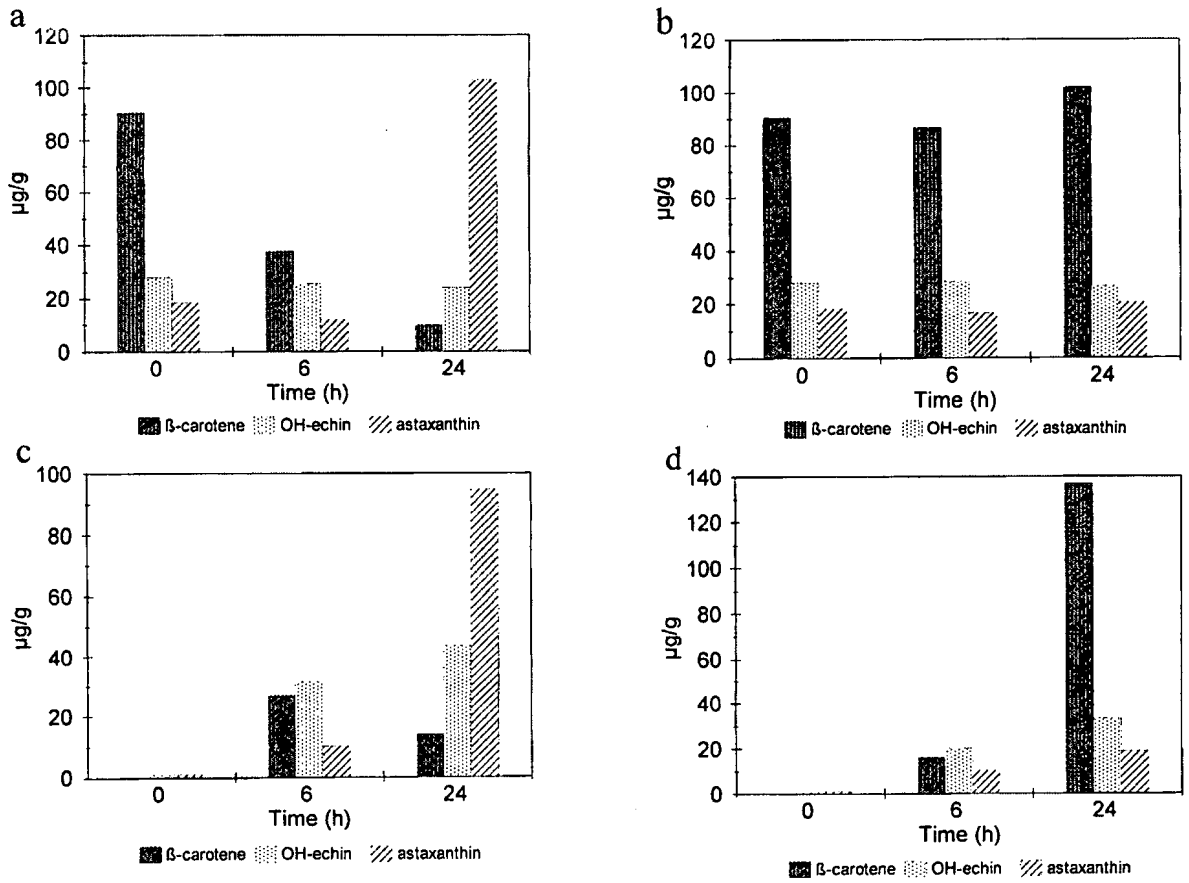

Figure 4. Carotenoid composition (µg/g biomass) of wild-type *X. dendrorhous* grown in the presence of diphenylamine (a and b with 10 µM, c and d with 60 µM). Time 0: before removal of diphenylamine and addition of cycloheximide (10 mM) when stated (b and c). Times 6 and 24: hours of incubation after removal of diphenylamine. Incubation temperature: 22°C.

was divided into two equal parts. After sedimenting the cells, one part was washed and reincubated in fresh media without inhibitor, the other part was not washed but also reincubated in fresh media in the presence of the same inhibitor at equal concentration as before; both cultures were maintained at 4°C. In the absence of inhibitor the culture re-established the normal carotenoid pattern as observed at 22°C. When nicotine was present in the fresh media the lycopene concentration remained constant without cyclizing to β-carotene. A different response was found with diphenylamine (10 µM). Figure 5a shows that even in the presence of inhibitor, β-carotene was converted to astaxanthin at 4°C. This did not happen when protein synthesis was inhibited by cycloheximide (Figure 5b). In order to test whether diphenylamine was metabolized at 4°C we incubated the washed cells from a culture of *X. dendrorhous* grown in the presence of nicotine (10 mM) with the filter-sterilized media derived from a culture at 4°C in the presence of diphenylamine. In this experiment the accumulated lycopene was converted mainly to β-carotene instead of astaxanthin, compared with the control in the absence of inhibitor (Figure 6).

DISCUSSION

In *X. dendrorhous* two possible biosynthetic pathways have been postulated for astaxanthin (Figure 1) where neurosporene is the branching point (Johnson and An, 1991). The most accepted pathway involves the cyclation of lycopene through γ-carotene yielding β-carotene probably by a

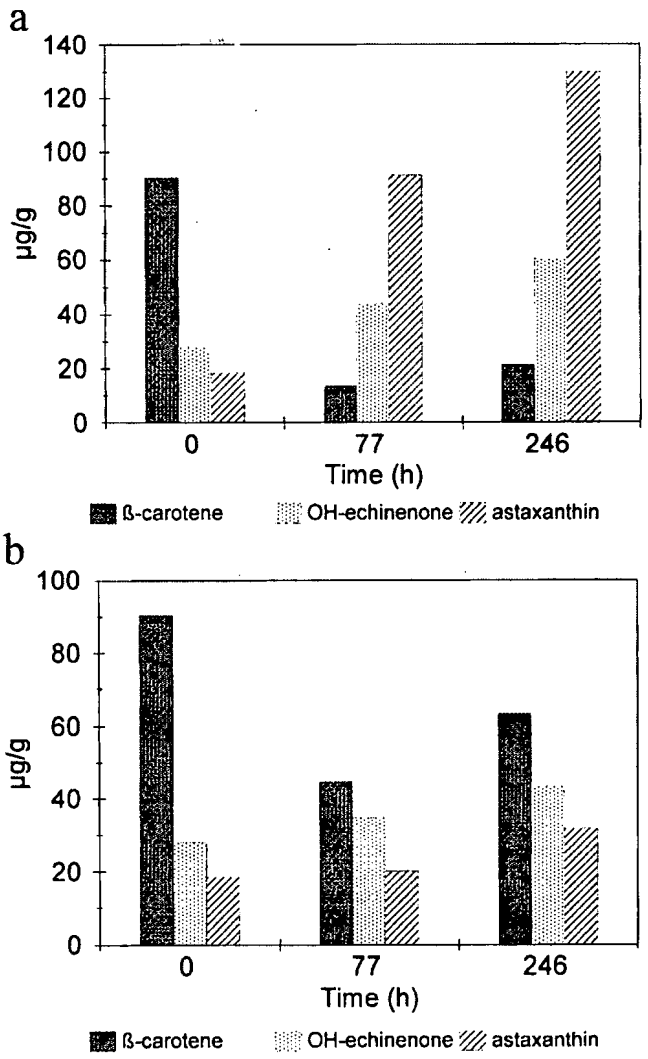

Figure 5. Carotenoid composition (µg/g biomass) of wild-type *X. dendrorhous* grown in the presence of diphenylamine (10 µM). Time 0: before removal of diphenylamine and addition of cycloheximide (10 mM) when stated (b). Times 77 and 246: hours of incubation after removal of diphenylamine. Incubation temperature after the washing step to remove diphenylamine: 4°C.

lycopene cyclase. This enzyme is inhibited by nicotine and according to our results removal of the inhibitor is sufficient to complete the reaction. *De novo* synthesis of protein is not necessary since the conversion is also observed when cycloheximide is added at inhibitory concentrations after removal of nicotine. Inhibition of carotenogenesis by diphenylamine was traced in various organisms to a block in the desaturation of phytoene (Candau *et al.*, 1991; Bejarano and Cerdaå-Olmedo, 1989). Two different inhibition targets were described by Sassu (1972) in *Dictyococcus cinnabarinus*, where similar to our results a low concentration of diphenylamine (10 µM) caused the accumulation of β-carotene and iso-zeaxanthin (not found in our experiments), while at high concentrations (50 µM) accumulation of phytoene occurred. Fan *et al.* (1995) found the same effect in the *cis*-astaxanthin-producing microalga *Haematococcus pluvialis* (30 and 60–90 µM). In *H,. pluvialis* the synthesis of

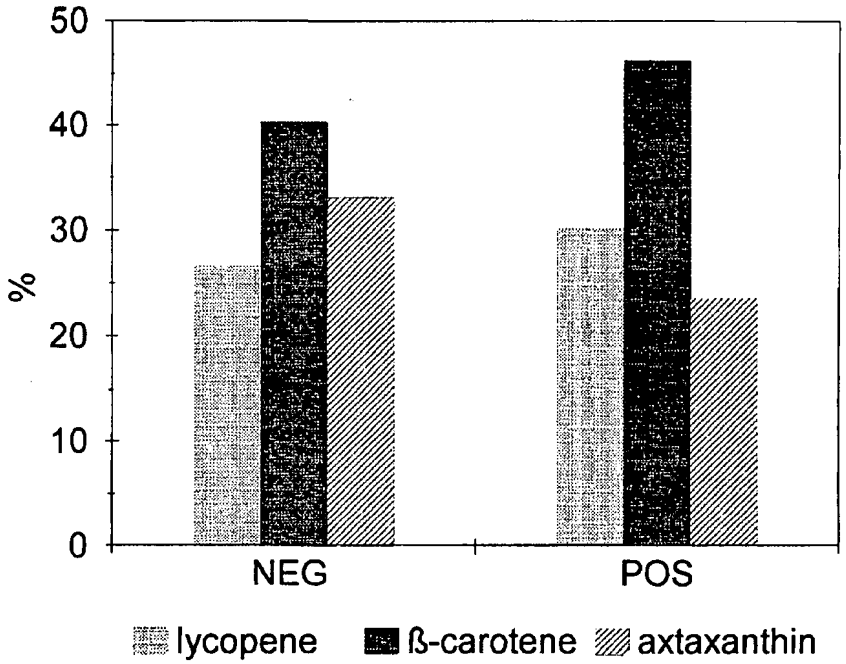

Figure 6. Astaxanthin, β-carotene and lycopene composition (%) of wild-type *X. dendrorhous* grown in the presence of nicotine (10 nM), followed by a second incubation with media coming from cultures grown in the presence of diphenylamine at 4°C (10 μM) (POS) and absence of diphenylamine at 4°C (NEG), after removal of the nicotine by a washing step. Incubation temperature after removal of nicotine: 22°C.

astaxanthin seems to be carried out by two different enzymes. The β-carotene ketolase (*bkt* gene) introducing the C-4 keto groups yielding the intermediate cantaxanthin has been cloned and expressed in *Escherichia coli* (Kajiwara *et al.*, 1995; Lotan and Hirschberg, 1995). In our study, the release of the inhibition by diphenylamine of the desaturation of phytoene did not require *de novo* protein synthesis, suggesting a competitive inhibition of the enzyme(s), in accordance with the *in vitro* effect in *Phycomyces* cell extracts, where the inhibition is also reversible (Clarke *et al.*, 1983).

In the presence of cycloheximide the oxidation step is still blocked, even after the removal of diphenylamine, indicating that the inhibition of the oxygenase step is not readily reversible. In *X. dendrorhous* the lack of cantaxanthin, the presence of hydroxyechinenone (Andrewes *et al.*, 1976) and the failure to produce mutants in any of the intermediate steps in the oxidation of β-carotene to astaxanthin (Calo *et al.*, 1995; Lewis *et al.*, 1990; Meyer *et al.*, 1993) leads to the hypothesis that only one enzyme carries out this conversion. The β-carotene ketolase from *H. pluvialis* and the C-4 keto group introducing activity from *X. dendrorhous* may have some kind of relation since both are inhibited by diphenylamine; however, the double inhibitory effects on carotenogenesis are not clear. Fan *et al.* (1995) discussed whether diphenylamine interacts direct or indirectly with the oxygenases. We determined here that after 4 days of incubation at 4°C the ability of β-carotene to inhibit oxidation persists in the culture media.

In conclusion, the inhibition by diphenylamine at low concentrations could be more complicated than a simple inhibition at the enzymatic level and could be part of a regulatory event at the gene level. In that case the increase in the carotenogenesis induced directly or indirectly by cold temperature conditions, could be at the same level and suppress the inhibition.

The release of the inhibition of the β-carotene oxidation by diphenylamine at 4°C needs *de novo* protein synthesis and is blocked by cycloheximide.

If two pathways are postulated for the biosynthesis of astaxanthin in *X. dendrorhous*, the increase of intermediates such as echinenone could demonstrate that the primary pathway is responsible for the synthesis of astaxanthin. Schroeder and Johnson (1995a) have concluded that only one pathway exists, the one originally proposed by Andrewes *et al.* (1976), and that other carotenoids found normally in lower concentrations, which do not fit into the bicyclic precursor pathway, may be enzymatically or chemically derived products formed in reactions of the intermediates with radicals. The protective action of the carotenoids against oxidative damage by different oxygen and peroxide radicals has been extensively demonstrated (Schroeder and Johnson, 1995b; Ducrey *et al.*, 1998).

If there is no alternative pathway, it remains unclear why HDCO accumulates in considerable amounts. Schroeder and Johnson (1995a) postulate that HDCO may also be an enzymatic degradation product of another xanthophyll.

The majority of the publications on *X. dendrorhous* do not include data on phytoene concentrations, with the exception of Girard *et al.* (1994) who measured it in a wild-type strain CBS 6938, as well as in several mutants. In all of them the concentration was very small (3–80 μg/g). Even in albino mutants where it comprises the end product, the concentration does not exceed 175 μg/g. Contrarily, Chun *et al.* (1992) isolated an albino mutant from strain CBS 5905 producing more than 2570 μg/g of phytoene, but in the parental strain as well as in fusion products, where the normal pigmentation was restored, phytoene was not detected. The phytoene concentration observed by Chun could reflect some kind of deregulation since the parental strain (CBS 5905) and the fusion product produced only 370 and 460 μg/g of total carotenoids, respectively.

We measured a large amount of phytoene (Table 1) in the wild-type *X. dendrorhous* NRRL Y-17269 growing under normal culture conditions at 22°C. The fact that the concentration of phytoene does not increase compared to a control when the astaxanthin synthesis is inhibited (diphenylamine 10 μM) but increases when lycopene instead of β-carotene (nicotine) accumulates and also in the presence of 60 μM-diphenylamine, could indicate that β-carotene plays a role in feedback regulation instead of astaxanthin. β-carotene, as a major end product, seems to regulate its own synthesis in *Phycomyces blakesleeanus* (Bramley and Mackenzie, 1988) by feedback inhibition on the step(s) before phytoene. While the biosynthetic pathway of carotenoids appears to be quite universal, the regulatory mechanisms are different. The fungi *Phycomyces*, *Blakeslea* and *Gibberella* respond in different ways to agents modifying carotenogenesis, such as visible light and various chemicals (Cerdaå-Olmedo, 1994). Also the induction mechanism at low temperatures remains unclear. It could involve the production of free radicals, which are reported to induce carotenogenesis in *X. dendrorhous* (Schroeder and Johnson, 1995a; Schroeder *et al.*, 1996).

Further studies are needed to determine the exact role β-carotene plays in the regulation of carotenogenesis in *X. dendrorhous* and if it is compatible with the hypothesis of feedback regulation by astaxanthin proposed by Schroeder and Johnson (1995a).

Our results indicate that the dehydrogenation step(s) of phytoene is a bottle neck in *X. dendrorhous* NRRL Y-17269 in the carotenoid biosynthesis. Improvement at this stage should lead to greater production of astaxanthin by this strain.

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